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Potential use of allicin (garlic, Allium sativum Linn, essential oil) against fish pathogenic bacteria and its safety for monosex Nile tilapia (Oreochromis niloticus)

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A study was conducted to investigate the antibacterial activities of allicin, one of the major essential oils of garlic, against some fish pathogenic bacteria as well as the toxic effect of allicin on monosex Nile tilapia (*Oreochromis niloticus*). Allicin 10% v/v solution in dimethyl sulfoxide (DMSO) was used in this study. The bacterial species investigated were *Aeromonas hydrophila, A. caviae, A. sobria, A. veronii,* and *Streptococcus iniae*. The minimum inhibitory concentrations (MICs) of allicin in DMSO against *Aeromonas hydrophila* was found to be 125 µg/mL, while ranged between 250–500 µg/mL for each of *A. caviae, A. sobria, A. veronii*. Interestingly, the MIC for *Streptococcus iniae* was 63 µg/mL. On contrary, allicin exists no bactericidal activity against any of the investigated bacteria. Allicin had high toxic effect on tilapia fingerlings while adult tilapia could tolerate it.

Global population increase and food demand have been motivating factors leading to additional expansion of intensive animal and fish production. Recently, many attempts advocated the expansion in fish farming to optimize the real need for animal protein. Concurrently, and as a result of fish farming and its intensification, many fish diseases appeared and spread particularly among cultured fishes (Essa and Khater, 1996; Shoemaker and Klesius, 1997). Some members of aeromonads and Streptococci have had increasing prominence as agents of fish disease with particularly severe losses among cultured and wild fishes (Austin, 1999).

The greatest attention was paid by researchers to aeromonads, in particular Aeromonas, and its biotroph in connection with epizootics among aquaculture that have become more frequent especially under fish breeding and intensification. In addition, the infections with members of *Aeromonas* caused large-scale spoilage and great economic losses of fish and fisheries products (Joseph *et al.*, 1991 and Kompanets *et al.*, 1992). Diseased fish displayed signs of hemorrhagic septicemia, namely hemorrhages on the body, intestine filled with bloody exudates, enlarged liver and spleen, and liquefied kidney. The causal agents of such outbreaks was identified as *Aeromonas hydrophila, A. caviae, A. sobria, A. veronii,* and, *A. jandaei* (Esteve *et al.*,1994; Esteve, 1995; Abu-El-Saad,1996; Ogara *et al*., 1998 and Austin, 1999; Pachanawan *et al.*, 2008).

Streptococcal infection is one of the more serious fish diseases caused by gram-positive bacteria. It has assumed considerable importance in recent years because of the high economic losses in both wild and cultured fishes. β-hemolytic, α-hemolytic, and non-hemolytic streptococci have been reported as causative agents of fish diseases (Al-Harbi 1994 and Yuasa *et al*., 1999; Musa *et al.*, 2009). Among β-hemolytic streptococci, *Streptococcus iniae* has been frequently reported associated with the mortality of tilapia species (Parera *et al*., 1994; Sugita, 1996). Streptococcosis signs in tilapia were lethargy, erratic swimming and dorsal rigidity, while in rainbow; there was septicemia and brain damage (Eldar *et al*., 1994).

To cope with the serious problems of the bacterial diseases outbreaks, various antibiotics are sometimes applied as treatments. Concurrently, the amount of antibiotics utilized in the treatment of fish diseases has increased because of intensive fish production increases. On the other hand, habitual use of antibiotics can leads to other problems such as, the risk of developing bacterial resistance, hazardous residues in fish flesh, and may also cause serious impact on aquatic ecosystems (Direkbusarakom *et al*., 1998; Agersøa *et al.*, 2007). Furthermore, the emergence of bacterial antibiotic resistance might be transmitting from aquaculture

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environments to humans and animals (Sapkota *et al*., 2008). For these reasons, many researchers have been investigating the use of antimicrobial safer compounds that have no harmful effects on fish, consumers and ecosystems (Disyaboot, 1975; Rath, 1990; Rach *et al*., 2000; Smith, 2001; Lunestad and Samuelsen, 2001; Hussein and Hassan 2004; Pachanawan *et al*., 2008).

Garlic (*Allium sativum* Linn.) and its constituents, has previously been examined for their antimicrobial activities (Yano *et al*., 2006; Sofia *et al*., 2007). However, little researches were carried out concerning these activities against fish pathogenic bacteria. It was indicated that the antibacterial activity of garlic resulted from thiosulfinates, particularly allicin, which is responsible for most of the antimicrobial activity, as well as its flavor and aroma (Ankri and Mirelman, 1999; Amagase *et al*., 2001; Harris *et al*., 2001).

This study was conducted to determine the antibacterial activities of allicin, the main essential oil of garlic, against four pathogenic strains of Aeromonads and one pathogenic strain of Streptococci. Tolerance tests were also conducted using young tilapias to determine its safety for use in aquaculture applications.

Materials and methods

Garlic essential oil. Allicin (diallyl disulfide, 99.9%), was obtained from Seven Seas Pure Chemical industries, Ltd., China. Allicin is poorly miscible in water, so dimethyl sulfoxide (DMSO) was chosen as a solvent for allicin. A 10% v/v solution in DMSO (Hussein and Hassan 2004; Hayet *et al*., 2009) was prepared and used as the original solution for the present experiments. At the levels tested, DMSO has no effect either on fish or tested bacterial strains (Hussein and Hassan 2004).

Bacterial strains. Five strains of fish pathogenic bacteria were obtained from different locations as shown in (Table 1). They include four strains of Aeromonads namely, *Aeromonas hydrophila, A. caviae, A. sobria, A. veronii* and one strain of *Streptococcus iniae.* The selected bacterial strains were isolated from outbreaks of bacterial diseases, purified, morphologically and biochemically classified, then kept for further experiments either in micro-bank tubes under (–80°C) and/or in semisolid agar at 4°C with regular subculture every three months. All tested bacterial strains were exposed to pathogenicity test before being used in this study. They were grown and maintained on brain heart infusion agar plates (BHIA, Oxide®) and were incubated at 25° C in all experiments within this study.

Fish. The toxic effect of allicin was determined on young fingerlings of Nile tilapia (*Oreochromis niloticus*) and adult ones. Live monosex Nile tilapia fingerlings having approximate average weights of 2 ± 0.5 g, and an approximate average length of 2 ± 0.5 cm, and adult Nile tilapia of approximate average weight of 35 ± 2.0 g and an approximate average length of 24 ± 2 cm were obtained from a private Tilapia hatchery at Beni-Suef Governorate, Egypt. Fingerlings were acclimatized up to four weeks in aerated 100 L aquaria, while the adult ones were acclimatized up to two weeks in 200 L fiberglass tanks. Both fingerlings and adult fish were fed a commercial feed (Geo-trad, El Asher men Ramadan, Giza, Egypt) in a powder form for the former and pellet ones for the later until 3 days prior to the experiment.

Allicin bacteristatic activity. The bacteristatic effect of the allicin solution was performed by an agar plate dilution method through incorporating the solution directly into the melted BHIA medium (Christofilogiannis, 2001; Hussein and Hassan 2004). Briefly, allicin solution was prepared at 10 times the desired final concentration and 1 mL of this solution was placed in sterile Petri dishes (20 X 80 mm). Then nine mL of melted medium was added with gentle shaking in order to distribute the allicin solution in the culture medium evenly. To determine the minimum inhibitory concentration (MIC), plates with serial dilutions of allicin solution were prepared beginning with the greatest concentration of 1000, 500, 250, 125, and 63µg/mL. Control plates containing the medium plus 1 mL DMSO in distilled water (DW) instead of allicin were also prepared. The prepared plates (duplicates) were inoculated with each tested bacterial strain, and then kept at 25°C. The growth of each bacterial strain was checked after 24 and 48 hours.

Table (1): Bacterial strains used for investigation of the antibacterial activity of allicin.

Isolate	Isolation Year	Host or source	Location	Species
BSV 0804	2008	Nile tilapia	Beni-Suef, Egypt	*Aeromonas hydrophila*
BJM 0005	2000	Red tilapia	Bali, Indonesia	*Aeromonas caviae*
BJM 9916	1999	Tilapia and common carp	Tigbauan, Philippines	*Aeromonas sobria*
BJM 9824	1998	Sockeye salmon	Hokkaido, Japan	*Aeromonas veronii*
B0035	2003	Tilapia species	Beni-Suef, Egypt	*Streptococcus iniae*

Allicin bactericidal activity. The bactericidal effect of allicin (garlic essential oil) was determined using a broth micro-dilution assay (Qaiyumi, 2007). The 96-well sterile micro-titer plates with U-bottom wells were prepared by dispensing into each well 160 µL of BHI broth. The first column of wells in micro-titer plate received a 20 µL of allicin solution at 10 times the desired final concentration starting with 10000 µg/mL. After mixing by pipetting, the mixtures (90 µL) were transferred to the next column of wells in a process of double-fold serial dilution until column number 10 to obtain final concentrations of 1000, 500, 250, 125, 63, 32, 16, 8, 4, 2 µg/mL. Columns number 11 and 12 were designated as controls with the former containing BHI broth with DMSO and the later contained only BHI broth. A suspension of each bacterial strain in sterile saline was prepared and adjusted to 1.5 X 10^8 CFU/mL, according to McFarland tube No.0.5. Then, 10 µL of each selected bacterial pathogen were inculcated into one wells row of the micro-plate and incubated at 25ºC for 24 hours.

The viability of the tested bacterial strains was determined by observing the growth appearance of the tested bacteria in comparison with the control ones and confirmed by plating 20 µL from each well onto BHI agar medium and observed over a period of 24 and 48 hours.

Tolerance test. One hundred and eighty monosex Nile tilapia (*Oreochromis niloticus*) fingerlings were divided into six groups with thirty fish per group. One liter of dechlorinated aerated water were placed in clean glass beakers and then the required amount of allicin solution was added to obtain 1000, 500, 250, 125, 63 and 0.0 (control) µg/mL. Fish were added to the glass beakers only after complete dissolving of allicin solution. Immediately after the fish were placed in the glass beakers, air stones were added. The water temperature was 22 ± 0.5°C and the exposure times were 15, 30, and 60 minutes. After elapsing of each exposure time, ten individuals from each fish group were removed from the beaker and placed in 30 X 15 X 20 cm aquaria supplemented with aeration. To examine the toxic effect of allicin on the adult tilapia, 180 randomly selected fish were divided into six groups with thirty individuals each. Each group was placed in 50 X 25 X 40 cm aquaria supplemented with aeration. Adult fish were treated with allicin concentrations and exposure times at the same manner as fingerlings ones. After elapsing of all exposure times, survivals of both treated fishes (fingerlings and adult Nile tilapia) were observed for 24 hours for any abnormalities that may develop. A fish was judged to be dead when opercular movements had completely ceased.

Results

Allicin bacteristatic activity. The antibacterial inhibitory effect of allicin solution varied considerably. For example, the MIC of allicin solution for *A. hydrophila,* was found to be 125µg/mL. On contrary, the MICs of allicin solution for *A. caviae, A. sobria,* and *A. veronii* were 500µg/mL, for each. However, *A. caviae, A. sobria* and *A. veronii*, expressed a very faint growth at allicin concentration of 250µg/mL. Interestingly, the MIC for *Streptococcus iniae* was 63 µg/mL (Table 2).

Allicin bactericidal activity. At all tested concentration levels, allicin solution did not kill or even inhibit the growth of the tested bacterial strains, when a 20 µL from each well were plated onto BHI agar medium and observed over a period of 24 and 48 hours. Further more, the growth on control plates seems to be the same on the tested ones.

Tolerance test. After all exposure times, 100% mortalities were detected among tilapia fingerlings exposed to 1000, 500, 250, and 125 µg/mL. On the other hand, tilapia fingerlings that exposed to 63µg/mL for all exposure times were recovered and survived. On the contrary, at all tested levels, all adult tilapia groups that were exposed to the previous allicin concentrations showed strong tolerance. In contrast, adult

tilapia groups that were exposed to allicin concentrations of 125 and 63 µg/mL for 15, 30, and 60 minutes experienced survival rates of 100%. In addition, adult tilapia groups that exposed to allicin concentration of 250 µg/mL for 15, 30, and 60 minutes showed survival rates of 100, 100, and 30 %, respectively. However, adult tilapia groups that exposed to allicin concentration of 500 µg/mL for 15, 30, and 60 minutes showed survival rates of 100, 10 and 0.0 %, respectively.

Moreover, all tilapia groups, except one, that were exposed to allicin concentration of 1000 µg/mL experienced 100% mortality. The exception was the tilapia group that was exposed to 1000 µg/mL for 15 minutes. Eventually, 100% of the fish in this group recovered and survived (Table 3).

Table (2): Bacteristatic effects of prepared allicin solution on selected fish pathogenic bacterial strains.

Species / isolate	Allicin, 10% in DMSO (µg/mL) [a]				
	1000	**500**	**250**	**125**	**63**
Aeromonas hydrophila	– [b]	–	–	–	+ [c]
Aeromonas caviae	–	–	± [d]	+	+
Aeromonas sobria	–	–	±	+	+
Aeromonas veronii	–	–	±	+	+
Streptococcus iniae	–	–	–	–	–

[a] concentration of allicin, [b](–) negative growth, [c] (+) positive growth, and [d] (±) faint growth

Table (3): Toxic effect of allicin on Nile tilapia fingerlings and adult ones.

Allicin concentration (µg/mL)	Exposure time (min)	Survival rate (%) at 22 ± 0.4°C	
		Adult.	Fingerlings
1000	15	100	0
	30	0	0
	60	0	0
500	15	100	0
	30	10	0
	60	0	0
250	15	100	0
	30	100	0
	60	30	0
125	15	100	0
	30	100	0
	60	100	0
63	15	100	100
	30	100	100
	60	100	100
Control	15	100	100
	30	100	100
	60	100	100

Discussion

The preceding results show that allicin, as one of the major essential oils of garlic, has a significant antibacterial activity (Table 2). More obviously, the presented results show that allicin *in vitro* inhibited the growth of the tested bacterial aeromonads at concentrations of 250 - 500 µg/mL, with the former had a weak bacteristatic effect while the latter showed a strong one. However, allicin at a concentration of 125µg/mL could inhibit the growth of *A. hydrophila* completely. These concentrations are comparable with those reported by Pachanawan *et al*., (2008), who found that water, 95% ethanol or ether extract of garlic powder at a concentration of 0.1mg/ml inhibits the growth of *A. hydrophila* in cultured media using swab paper disc method. The difference between the present results and those of Pachanawan *et al*., (2008) may be due to presence of some antibacterial impurities in garlic extract. In addition, it was reported that the antibacterial activity of garlic oil (undiluted form) is 200 times greater than garlic powder and 900 times

the strength of fresh garlic (Helou and Harris, 2007). According to a review by Ankri and Mirelman (1999), *Streptococcus β hemmolyticus*, *Pseudomonas aeruginosa* and *Enterococcus faecium* were found to be resistant to allicin and this resistance may be due to presence of hydrophilic capsular or mucoid layers prevent the penetration of allicin into the bacteria. On contrary, according to the finding of this study allicin at a concentration of 63 µg/mL could inhibit *Streptococcus iniae*. Similarly, Alsaid *et al.*, (2010), showed that the MIC of 10% water extract of garlic against *Streptococcus agalactiae* was 2.5 mg/mL.

As a result within our study, allicin at concentrations of 1000, 500, 250, 125, 63, 32, 16, 8, 4, and 2 µL/ml could not kill the tested bacterial strains. These differences may be due to the character and level of antimicrobial agents present in allicin and its mode of action. These results were comparable with those reported by Rattanachaikunsopon and Phumkhachorn (2009), who examined the mode of action of four herbal essential oils against *Lactococcus garvieae*, and they found that *L. garvieae* cells inhibited by ginger oil and holy basil oil have the ability to re-grow when they transferred into fresh BHI broth 24 hour after transfer, indicating the bacteriostatic mode of action of the oil. This may explain why the tested bacterial strains re-grow when they were plated on fresh BHI plates.

Based on our knowledge, little work has been carried out on the toxic effect of allicin on Nile tilapia (adult and fingerlings). Our results revealed high toxicity of allicin for tilapia fingerlings at the tested levels, however, adult ones showed tolerance to allicin (Table 3). This may indicate that the age of treated fish with allicin is an important factor. Therefore, investigations of the factors influencing allicin toxicity are needed. However, the potential was evident to use such compound against warm water fishes such as adult tilapia spp.

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إمكانية استخدام الأليسين (زيت الثوم الأساسى) ضد البكتريا الممرضة للأسماك ودرجة أمان استخدامه فى أسماك البلطى النيلى أحادى الجنس

تم عمل هذه الدراسة بغرض الاستقصاء عن نشاط الأليسين المضاد للبكتريا (زيت من المكونات الرئيسية للثوم) باستخدام بعض البكتريا الممرضة للأسماك وأيضا معرفة التأثير السمى للأليسين لأسماك البلطى النيلى أحادية الجنس. تم استخدام محلول الأليسين ١٠% (حجم/ حجم) فى مذيب الديميثيل سلفوكسيد. تم استخدام بعض العترات البكتيرية الممرضة للأسماك فى هذه الدراسة وهى إيروموناس هيدروفيللا ، إيروموناس كافيى ، إيروموناس سوبريا ، إيروموناس فيرونى ، وعترة المكور سبحي آيني. وقد تبين أن أدنى تركيزات مثبطة من الأليسين لعترات الإيروموناس هيدروفيللا ١٢٥ ميكرو جرام /ملل بينما تراوحت أدنى تركيزات مثبطة من الأليسين لكل من الإيروموناس كافيى ، والإيروموناس سوبريا ، والإيروموناس فيرونى من ٢٥٠ - ٥٠٠ ميكرو جرام /ملل. ومن المذهل ، ان التركيز المثبط لعترة الاستربتوكس آيني هو ٦٣ ميكرو جرام /ملل. وعلى النقيض ، فإن الأليسين لم يظهر أى تأثير بكتيرى قاتل ضد أى من العترات البكتيرية المستخدمة. بالنسبة لتأثير الأليسين على الأسماك المستخدمة تبين أن له تأثير سمى قوى على اصبعيات أسماك العائلة البلطية بينما استطاعت أسماك العائلة البلطية البالغة مقاومة هذا التأثير عند التركيزات المستخدمة فى هذه الدراسة.